Relaxed selection is a precursor to the evolution of phenotypic plasticity

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Phenotypic plasticity allows organisms to produce alternative phenotypes under different conditions and represents one of the most important ways by which organisms adaptively respond to the environment. However, the relationship between phenotypic plasticity and molecular evolution remains poorly understood. We addressed this issue by investigating the evolution of genes associated with phenotypically plastic castes, sexes, and developmental stages of the fire ant Solenopsis invicta. We first determined if genes associated with phenotypic plasticity in S. invicta evolved at a rapid rate, as predicted under theoretical models. We found that genes differentially expressed between castes of S. invicta castes, sexes, and developmental stages all exhibited elevated rates of evolution compared with ubiquitously expressed genes. We next investigated the evolutionary history of genes associated with the production of castes. Surprisingly, we found that orthologs of caste-biased genes in S. invicta and the social bee Apis mellifera evolved rapidly in lineages without castes. Thus, in contrast to some theoretical predictions, our results suggest that rapid rates of molecular evolution may not arise primarily as a consequence of phenotypic plasticity. Instead, genes evolving under relaxed purifying selection may more readily adopt new forms of biased expression during the evolution of alternate phenotypes. These results suggest that relaxed selective constraint on protein-coding genes is an important and underappreciated element in the evolutionary origin of phenotypic plasticity.


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We further used *S. invicta* to investigate the molecular evolution of genes with expression bias between sexes and developmental stages. Hymenopterans are particularly well suited for such investigations because sex is determined by differences in ploidy level (hymenopteran males are typically haploid and females are diploid (28)) rather than by sex chromosomes. Moreover, hymenopterans undergo complete metamorphosis and display dramatic phenotypic differences among developmental stages (as in other holometabolous insects (12)), hence providing an additional comparison of alternate developmental phenotypes. Our analyses of castes, sexes, and developmental stages in *S. invicta* facilitate a direct comparison of the molecular evolution of loci associated with diverse forms of biased gene expression.

**Results and Discussion**

**Gene Expression Bias Represents a Key Factor Associated with the Rate of Protein Evolution.** Microarray analyses conducted on whole bodies of adult and pupal males, queens, and workers of the fire ant *S. invicta* (Fig. 1A) revealed that many genes were differentially expressed between age-matched female castes, sexes, and developmental stages (15) (Fig. 1B). We investigated if gene expression bias was associated with the rate of gene evolution by analyzing coding sequences from the ants *S. invicta* (29), *Pogonomyrmex barbatus* (30), and *Linepithema humile* (31).

We found that the rate of protein evolution (measured as the ratio between the rate of nonsynonymous and synonymous substitutions (dN/dS)) in *S. invicta* was significantly correlated with two principal components representing the pooled effects of gene expression bias (PC1) and overall gene expression level (PC3) (32) (Fig. 2 and Fig. S1). The strengths of correlations between protein evolutionary rates and PC1 and PC3 were similar but in opposite directions (Fig. 2B). Thus, diverse forms of gene expression bias, when taken together, contribute to protein evolutionary rate variation at levels comparable to overall gene expression level in *S. invicta*. This demonstrates that gene expression bias among individuals and developmental stages is a key factor (32–34) associated with the rate of protein evolution.

The correlations between the degree of gene expression bias and protein evolutionary rate were significant for all three instances of alternate phenotypes examined (Fig. S2). Furthermore, there was an approximately proportional relationship between the rate of protein evolution and degree of gene expression bias in all three cases, with the highest dN/dS value attributable to genes with greatest expression bias among morphs (Fig. 3A). The association between evolutionary rate and gene expression bias was also approximately additive; genes that showed bias in multiple different contexts (i.e., castes, sexes, developmental stages) tended to evolve more rapidly than genes that showed bias in only a single context (Fig. 3B and C).

We conducted a separate analysis of the relationship between gene expression bias among castes and rate of protein evolution in the honeybee *A. mellifera*, which evolved sociality in a lineage independent from ants. Previously, we analyzed gene expression data from *A. mellifera* queen and worker brain tissue (35) to highlight differences in evolution between queen- and worker-biased genes (20). In a reanalysis of these data, we found that, as in *S. invicta*, the overall degree of differential gene expression between *A. mellifera* queens and workers was positively associated with the rate of gene evolution (Fig. S3). This finding further supports the view that biased gene expression is associated with an increase in protein evolutionary rate in polyphenic social insects.

**Purifying Selection Explains Most Variation in Protein Evolutionary Rate.** Variation in the rate of protein evolution may reflect differences in the strengths of positive selection or purifying selection. To understand the relative contributions of these different modes of selection better, we conducted several analyses to detect signatures of positive selection during *S. invicta* protein evolution.

These analyses suggested that positive selection was not dominant in shaping overall rates of protein evolution. Only 3 of 1,104 *S. invicta* genes with expression data and orthologs in other taxa exhibited clear evidence of positive selection when averaged across all codons (dN/dS > 1); for the remaining genes, mean dN/dS = 0.122 (SEM ± 0.003); Table S1). Four additional genes (of a set of 365 as discussed in Methods) exhibited a robust signature of lineage-specific positive selection in *S. invicta* according to consensus results from branch-site tests (36) based on two sequence-alignment methods (37) (Methods and Table S2). Overall, the paucity of genes exhibiting strong signatures of positive selection suggests that the observed correlations between gene expression bias and protein evolutionary rates were shaped largely by variation in the strength of purifying selection (33, 38).

To explore this issue further, we compared sequence polymorphism within *S. invicta* with sequence divergence between the *S. invicta* lineage and the outgroup taxa *P. barbatus* and *L. humile*. There were 381 genes with identified SNPs in *S. invicta*, sequence divergence data among taxa, and gene expression data in *S. invicta*. McDonald–Kreitman tests (39) were used to investigate the type of selection operating on these 381 genes, partitioned according to different levels of expression bias. We detected strongly significant (*P = 3.4e-9*) purifying selection in a pooled analysis of 223 genes showing low levels (i.e., less than 2-fold) of expression bias. There was also significant (*P = 0.048*) purifying selection detected in a pooled analysis of the 158 genes with greater than twofold expression bias in one or more contexts (Table S3). Thus, although it is likely that positive selection and demographic effects (40) contributed to some extent to the rate of molecular evolution, our data suggest that variation in protein evolutionary rate (Figs. 2 and 3) was largely driven by variation in the strength of purifying selection (33, 38). Moreover, the strength of purifying selection, as measured by the direction of selection (41), was stronger in genes not differentially expressed among morphs than in genes exhibiting differential expression (Table S3). This suggests that genes with biased expression evolved at elevated rates relative to unbiased genes, predominantly because purifying selection was weaker.
Rates of Protein Evolution and Different Phenotypic Forms. The rate of evolution of both queen- and worker-biased genes in S. invicta adult and pupal stages was higher than the rate of evolution of unbiased genes. However, the difference was significant only for the queen-biased genes (20) (Figs. S4 and S5C). Theory predicts that genes expressed in workers but not in queens should experience a relaxation of purifying selection relative to genes expressed in queens but not in workers, because selection operates indirectly on the sterile worker caste (42). However, our results show that worker-biased genes did not evolve faster than queen-biased genes. Moreover, the rates of evolution for worker- and queen-biased genes did not differ substantially from those of sex- or developmentally biased genes (Fig. S4). There are at least two possible nonmutually exclusive explanations for these results. First, indirect selection acting on workers or colony-level traits in social insects may not be substantially weaker than selection acting on other types of conditional traits. Second, the lack of a higher rate of evolution of worker-biased genes may stem from the fact that none of these genes were exclusively expressed in workers (17, 42).

In the case of development, adult-biased genes disproportionately contributed to an increase in protein evolutionary rates relative to unbiased genes (although pupal- and adult-biased genes did not differ significantly from one another in evolutionary rate; Fig. S4). Furthermore, little difference was observed between the rates of evolution of male- and queen-biased genes in S. invicta. This stands in contrast to results from many studies of sex-biased gene expression, where male-biased genes typically have been the primary driver of increased evolutionary rates associated with sex (10, 43).

Fast-Evolving Genes Were Preferentially Recruited in the Evolution of Social Insect Castes. We investigated whether the elevated rate of protein evolution observed for differentially expressed genes occurred before (19, 24) or after (17, 22, 23) the evolution of gene expression bias associated with phenotypic plasticity. To address this issue, we examined the evolution of proteins following the independent evolution of castes in ants and bees and in a parallel nonsocial lineage. We first identified 3,705 three-way orthologs of S. invicta, A. mellifera, and the nonsocial jewel wasp Nasonia vitripennis. In total, 1,050 of these orthologs were also associated with gene expression data from S. invicta castes (15).

Remarkably, the rate of evolution of genes in the N. vitripennis lineage was strongly associated with expression bias of the S. invicta orthologs (Fig. 4B). This result was further supported by an analysis of 1,289 orthologs (of the 3,705) for which expression data from A. mellifera castes were available (35). As was the case in S. invicta, the degree of caste bias of genes in A. mellifera was significantly associated with the rate of evolution of orthologs in N. vitripennis (Fig. 4C). Overall, these results support the view that rapid rates of protein evolution preceded biased gene expression associated with the evolution of castes and that fast-evolving genes were therefore recruited into the process of caste differentiation.

Did Relaxed Selection Precede the Evolution of Biased Gene Expression in Social Insects? Our results reveal that fast-evolving genes were preferentially recruited into caste-biased gene expression in both S. invicta and A. mellifera. This pattern could arise if genes under relaxed selective constraints were more likely to be adopted into mechanisms underlying the expression of alternate phenotypes. However, it is also possible that these genes already exhibited an elevated expression bias, among tissues (44) or sexes (10), for example, in the nonsocial ancestor of honey bees and ants. Indeed, a link between sex bias and tissue specificity has been observed in several taxa (24, 45), suggesting that tissue specificity and expression bias among morphs may be linked. If genes with bias in expression among tissues or between sexes are more likely to become differentially expressed in other contexts, social insect castes may have evolved largely by recruiting genes that were already involved in some type of phenotypic differentiation.

Unfortunately, the lack of data on gene expression in ancestors of A. mellifera and S. invicta makes it impossible to test whether tissue-specific or sex-biased genes have preferentially adopted caste-biased expression. However, it is possible to test whether the association between caste bias and sequence divergence is influenced by tissue specificity or sex bias in extant taxa. Such an analysis revealed that the observed correlations between caste-biased gene expression and protein evolutionary rate remained significant when controlling for either the degree of expression breadth in A. mellifera (46) or the sex- and developmentally biased gene expression in S. invicta (SI Text and Fig. S3). Although supporting the idea that tissue and sex specificity are not the main factors accounting for fast-evolving genes being preferentially adopted during the process of caste differentiation, the current analyses do not allow one to rule out
the possibility that caste-biased genes displayed some other type of expression bias in ancestral populations, which could have contributed to the observed relaxation of purifying selection.

**Concluding Remarks.** Our results suggest that genes with differential expression among alternate phenotypic forms evolve relatively rapidly because they are under relaxed selective constraint. Importantly, this association appears to stem primarily from fast-evolving genes being recruited in the process of phenotypic plasticity rather than from observed forms of gene expression bias themselves leading to relaxed purifying selection. Although relaxed selective constraint generally implies a diminished contribution to individual fitness at any given time point (e.g., 38), this lack of constraint may also facilitate specialized adaptation (18, 19, 47). In this manner, genes with limited selective constraints may play a particularly important role in the origin of phenotypic plasticity and the subsequent elaboration of specialized condition-specific phenotypes.

**Methods**

**Gene Sequences.** The following official gene sets (OGSs) were used in our analyses: S. invicta OGS 2.2.0 (29), P. barbatus OGS 1.1.3 (30), L. humile OGS 1.1 (31), Harpegnathos saltator OGS 3.3 (48), A. mellifera OGS 1 (49), Nasonia vitripennis OGS 1.2 (50), and Drosophila melanogaster flybase release 5.21.

**Ortholog Determination.** Pairwise orthology between proteins from each species was assigned based on reciprocal BlastP (54) hits with an E value cutoff of $E < 1e-10$. BlastP output was then parsed with a custom BioPerl script (55). Pairwise reciprocal best hits and orthogroups with three-way shared orthology and four-way shared orthology were assigned using custom Perl scripts. A total of 4,839 three-way orthologs between S. invicta, P. barbatus, and L. humile were found (5,398 with informative evolutionary rate data after filtering). A total of 4,839 three-way orthologs between S. invicta, A. mellifera, and N. vitripennis were found (3,705 with informative evolutionary rate data after filtering). A total of 5,476 four-way orthologs among the ant taxa were found (4,248 with informative evolutionary rate data after filtering).

**Sequence Alignment.** MUSCLE 3.8.31 (56) was used to generate protein alignments for each orthologous group with default settings. PAL2NAL v13 (57) was then used to back-translate codon alignments from protein alignments and Gblocks 0.91b (58) was used to extract confidently aligned regions. To confirm branch-site tests of positive selection, a separate alignment procedure (37) was undertaken. In this case, PRANK (59) (release 100802) was used to generate codon alignments with default settings.

**Evolutionary Rates.** PhyML 3.0 (60) was used to generate maximum likelihood phylogenies from codon alignments for each orthologous group with the “HKY85” nucleotide substitution model. PAML 4.4b (61) was then used to estimate $dN$ and $dS$ and $dN/dS$ for each orthologous group using a phylogenetic tree produced by PhyML as input and the “$F_3 \times 4$” codon frequency model.

For three-way orthologs between S. invicta, P. barbatus, and L. humile and three-way orthologs between S. invicta, A. mellifera, and N. vitripennis, substitution rates were averaged across all codons for a given protein (NSites $= 0$) with free $dN/dS$ ratios for each branch (model = 1). Three genes with evidence of positive selection ($dN/dS > 1$) in S. invicta were identified utilizing this approach. In each case, $dS$ was zero and $dN/dS$ values were infinite. These three genes were eliminated from further analysis to avoid skewing means but are summarized in Table 51. In our three-way comparison of S. invicta, P. barbatus, and L. humile, the mean $dS$ value for the S. invicta branch was 0.36 (SEM $\pm 0.006$; maximum $dS = 2.12$), indicating that synonymous sites have not been saturated by multiple substitutions and analysis of $dN/dS$ is appropriate. In our three-way comparison of S. invicta, A. mellifera, and N. vitripennis, the mean $dS$ value for the S. invicta branch was 3.94 (SEM $\pm 0.191$), indicating widespread synonymous site saturation. We therefore only used $dN$ for comparing these more divergent taxa.

**Fig. 3.** Relaxed selection is ubiquitously associated with gene expression bias in the fire ant S. invicta. (A) Protein evolutionary rate $dN/dS$ for genes showing varying levels of differential expression between sexes, castes, and developmental stages. (B) Overlap of genes with greater than twofold difference in expression between sexes, castes, and developmental stages. (C) Protein evolutionary rate for each section of the Venn diagram in B compared with all genes exhibiting less than twofold expression difference between sexes, castes, and developmental stages. Means with 95% confidence intervals are plotted in A and C, the text in bars denotes the number of genes in each bin, and significance was determined by Kruskal–Wallis tests.

**Fig. 4.** Evolutionary histories of genes with biased expression. (A) Phylogeny based on mean $dN$ for 3,705 orthologous groups, including A. mellifera, S. invicta, and N. vitripennis. Gray lines denote the independent evolution of sociality in S. invicta and A. mellifera. (B) Spearman’s rank correlations between the level of caste bias of genes (differential expression between queens and workers) in S. invicta and dN ($n = 1,050$ genes). (C) Spearman’s rank correlations between the level of caste bias of genes in A. mellifera and dN ($n = 1,289$ genes).

Figure 4. Evolutionary histories of genes with biased expression. (A) Phylogeny based on mean $dN$ for 3,705 orthologous groups, including A. mellifera, S. invicta, and N. vitripennis. Gray lines denote the independent evolution of sociality in S. invicta and A. mellifera. (B) Spearman’s rank correlations between the level of caste bias of genes (differential expression between queens and workers) in S. invicta and $dN$ ($n = 1,050$ genes). (C) Spearman’s rank correlations between the level of caste bias of genes in A. mellifera and $dN$ ($n = 1,289$ genes).
To test for further evidence of positive selection, we used the branch-site test of positive selection (branch-site model A, test 2) (36), which uses likelihood ratio tests to detect positive selection on a small number of sites along a specific lineage. For this test, we used MUSCLE alignments of four-way orthologs among the ants S. invicta, P. barbatus, L. humile, and H. saltator to construct phylogenetic trees using PhyML as before. S. invicta was set as the foreground branch, with two dN/dS ratios for branches (model = 2). For the alternative model, dS0 was estimated from the data (fix omega = 0, omega = 1), whereas the null model fixes dS0 at 1 (fix omega = 1, omega = 1) as described in PAML documentation and sample files (61). The log-likelihoods for the null and alternative models were used to calculate a likelihood ratio statistic, which was then compared against the $X^2$ distribution (with a critical value of 3.84 at a 5% significance level) (61). Positive values were Bonferroni-corrected according to the number of tests of significance performed ($n = 861$; $\omega_0 = 5.8\cdot5$ used as the threshold for significance). This highly conservative threshold was imposed because of the documented occurrence of false-positive results in branch-site tests of positive selection as a result of alignment errors (37). To control better for the influence of alignment errors, PRANK codon alignments were used as input in an alternative analysis of positive selection (37). For analysis of PRANK codon alignments, a Bonferroni cutoff of 1.4e-4 was used ($n = 365$). The consensus set of significant genes was then taken. Forty of 861 orthologs with MUSCLE alignments in our dataset retained a significant signal of positive selection on the S. invicta branch, whereas 5 of 365 orthologs with PRANK alignments in our dataset retained a significant signal of positive selection on the S. invicta branch. The consensus set consisted of four proteins (Table S2).

In each analysis of evolutionary rates, only genes with a length of $\geq 100$ codons analyzed by PAML were used in our results. For analyses of evolutionary rates using four-way orthologs, only ortholog groups exhibiting the most common phylogeny topology were used. Phylogeny topology, foreground branch specification, branch-specific substitution rates, and log-likelihoods were processed and parsed using custom Perl scripts.

### Polymorphism Analysis

Sequence reads from normalized S. invicta cDNA libraries were accessed from the National Center for Biotechnology Information Sequence Read Archive, including those generated from 100 heads of workers and queens from the United States (accession nos. SRR0600104 and SRR0600105) and those generated from two male each from 12 colonies in Argentina (accession nos. SRR060008, SRR060009, and SRR060010). Reads were assembled to a subset of OGS coding sequences with the highest possible quality rank (discussed in section on S. invicta array probe to genome mapping) using SSAHA2 with only uniquely high-scoring matches with 98% identity accepted (62). SAMtools (63) was used to assess variant sites utilizing "mpileup" and "bcftools." The SAMTools Perl script "vcfutils.pl" was then used to filter variants for an rms quality value of 20, a minimum read depth of 4, and a distance of at least 10 bases from a gap. Alternate coding sequences with alternate alleles substituted were then generated using a custom Perl script. We used PAML to calculate non-synonymous and synonymous substitutions on the branch in the three-way comparison of ants described above.

### S. invicta Gene Expression

Gene expression measures were obtained using custom cDNA microarrays (64, 65). We estimated the relative expression of each clone for whole-body age-matched adult and pupal queens, workers, and males using the Bayesian approach implemented in the program Bayesian Analysis of Gene Expression Levels (BAGEL) (66) as described previously (15). This BAGEL expression level was used to determine all log-transformed ratios of gene expression among phenotypic groups. We used the absolute value of log2-transformed pairwise BAGEL expression ratios to measure the degree of sex bias in expression (male vs. queen) and caste bias in expression (queen vs. worker) for adults and pupae separately. To measure developmental bias, the absolute value of the log2-transformed ratio of summed adult male, worker, and queen BAGEL values vs. summed pupal male, worker, and queen BAGEL values was taken.

The overall gene expression level for each of the array clones, which was not included in prior analysis, was calculated as follows. For each hybridization experiment, gene expression level was estimated for each clone as the ratio between its net signal intensity and the average net signal intensity across clones. In particular, for each hybridization experiment, $h$, we assigned to each clone, $c$, a relative expression $E_h^c$ where $n$ is the number of S. invicta good-quality clones (as flagged in GenePix, Axon Instruments, Foster City, CA) and F and B are, respectively, the foreground and background signal intensities for either the green or red channel, depending on the labeling of the sample. Then, the overall gene expression level for each clone was calculated across all k hybridizations as

$$E_h = \sum_{i=1}^{n} E_h^i$$

### S. invicta Array Probe to Genome Mapping

Sanger EST sequences of array clones were mapped to S. invicta genome scaffolds (assembly Si.grnF) using GMAP version 2010-03-09 (67). The best fit for each EST sequence was taken according to the genomic coordinates with the highest percent identity, and only ESTs with $\geq 95\%$ identity and 50% coverage were retained. Genome coordinates and strand information were extracted for each S. invicta gene from the OGS 2.2.0 general feature format (GFF) file, which was subsequently used to map ESTs to genes using a custom Perl script.

The mean relative BAGEL expression level and the overall gene expression level of duplicate spots on the array were first calculated, and the aggregate function was then used to take the mean of expression values for all clones that mapped to a given gene. This procedure resulted in gene expression measures for 2,088 unique genes (represented by 4,624 array clones). We filtered our dataset to include only S. invicta genes with proteins represented by a single cDNA and the highest possible quality rank (as indicated by the tag “quality = 5”).

### S. invicta Gene Characteristics

Normalized Cpg dinucleotide content acted as a metric of levels of DNA methylation and was calculated as described previously (68) (Table S4). Third-codon position synonymous guanine-cytosine (GC) content and the frequency of optimal codons (Fop) were calculated using CodonW (http://codonw.sourceforge.net). Fop was calculated with automatic detection implemented to use the top 5% genes (in terms of bias in codon use). Coding sequence length and intron counts were calculated from the OGS 2.2.0 GFF file using a custom Perl script.

### A. mellifera Gene Expression

Data on gene expression bias between brains of queens and workers in A. mellifera were obtained from a study by Grozinger et al. (35). Data on expression breadth among tissues in A. mellifera were obtained from a study by Forét et al. (46) and processed as described previously (69). RNA-Seq data on expression levels in A. mellifera whole-body workers were obtained from a study by Zemach et al. (70).

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Correlates of Protein Evolutionary Rate (dN/dS) in *S. invicta*. Analysis of potential evolutionary rate determinants (1–3) in *S. invicta* revealed that gene expression level was negatively correlated with dN/dS (Fig. S2F), as is the case in diverse taxa (4). In addition, coding sequence length was positively correlated with dN/dS (1) and intron number was negatively correlated with dN/dS (5), as observed in other taxa (1, 5). Interestingly, normalized CpG dinucleotide content, a measure of CpG depletion that is negatively correlated with DNA methylation in *S. invicta* (6), was positively correlated with dN/dS (Fig. S2F) and was largely decoupled from guanine-cytosine (GC) content and expression level (Table S4). Thus, methylated genes may be under greater functional constraint in *S. invicta* compared with unmethylated genes (3, 7). As is the case with the honey bee (8), GC content and codon use were tightly linked in *S. invicta* (third codon position synonymous GC content vs. Fop Spearman rank correlation = −0.996; Table S4).

Relationship Between Caste Bias and Protein Evolution in the Context of Other Forms of Conditional Gene Expression in *S. invicta*. We analyzed data describing tissue specificity of gene expression in *A. mellifera* (9) to determine whether the association between relaxed selection and caste-biased gene expression is driven by differences in pleiotropic constraints associated with tissue specificity (10). Our analyses revealed that the association between rates of protein evolution and caste-biased gene expression in *A. mellifera* was not weakened when controlling for tissue specificity in six tissue types (Spearman’s partial correlation between *A. mellifera* dN from Fig. 4A and caste bias, when controlling for tissue specificity = 0.120, *P* = 3.94e-5; *n* = 1,164; Fig. S3). Furthermore, the association between rate of protein evolution and caste-biased gene expression in *S. invicta* remained significant when controlling for sex bias and developmental bias (Spearman’s partial correlation between *S. invicta* dN from Fig. 4A and adult caste bias, when controlling for adult sex bias and developmental bias = 0.113, *P* = 0.0002; *n* = 1,050). This result is consistent with a scenario in which the observed correlation between caste bias and the rate of protein evolution is not explained by other forms of conditional gene expression.

dS and Caste in *S. invicta*. In the case of sex differences and developmental differences, dN was the primary driver of signal for increased dN/dS relative to unbiased genes (Fig. S5). Although dN is also associated with caste differences, dS was significantly lower for caste-biased genes relative to unbiased genes in both the pupal and adult stages (Fig. S5). Thus, caste-biased genes may have lower mutation rates or be subject to increased selection on synonymous codon use relative to unbiased genes.

Fig. S1. Principal component analysis of gene expression measures and evolutionary rates in *S. invicta*. Bars represent the relative contribution of each gene expression measure to each principal component when normalized by the Spearman’s rank correlation coefficient of each principal component with *S. invicta* dN and dS. The composition of principal components is illustrated in Fig. 2A.
Fig. S2. Correlations between morph-biased gene expression, protein evolutionary rate dN/dS, gene expression level, normalized CpG dinucleotide content (CpG o/e, a negative correlate of DNA methylation), third codon position synonymous GC content (GC3s), coding sequence (CDS) length, and intron count in S. invicta. Correlations between dN/dS and measures of (A) adult and (B) pupal sex-biased gene expression, (C) adult and (D) pupal caste-biased gene expression, and (E) developmentally-biased gene expression are each significant, as are partial correlations. (F) Correlations between dN/dS and several S. invicta gene characteristics. Black bars represent Spearman’s rank correlations. Gray bars represent partial correlations controlling for all other variables. Solid outlines on bars indicate $P < 0.05$ and wavy outlines indicate $P > 0.05$. 

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Fig. S3. Relationship between protein evolutionary rate, caste bias, gene expression level, and gene expression breadth in the honey bee *A. mellifera*. Branch-specific dN was determined for a three-species phylogeny between *A. mellifera*, *S. invicta*, and *N. vitripennis*, as in Fig. 4A. A total of 1,152 ortholog groups were analyzed with brain gene expression measures among castes (11), worker whole-body gene expression levels according to RNA-seq analysis (12), and the number of tissues with observed expression (ranging from 1 to 6) (9). Bars represent Spearman’s rank correlations and partial correlations.

Fig. S4. Differences in relaxation of selective constraint according to phenotype in *S. invicta*. Genes with greater than twofold expression difference, categorized according to the phenotypic group (significance determined by Bonferroni-corrected Wilcoxon signed-rank tests). Means with 95% confidence intervals are plotted; the text in bars denotes the number of genes in each bin.
Fig. S5.  dN and dS in relation to differential gene expression in S. invicta.  (A) dN denotes rates of nonsynonymous substitution.  (B) dS denotes rates of synonymous substitution.  (C) Dissection of the unique properties of caste-biased genes with respect to dN and dS.  Significance denotes results of Wilcoxon rank sum tests, subject to Bonferroni correction in C and D.  Means with 95% confidence intervals are plotted.

Table S1. Genes under positive selection as determined by substitution rates averaged over all codons in a given gene

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<td>2</td>
<td>0</td>
<td>∞</td>
<td></td>
<td>None</td>
<td>Drosophila melanogaster mago nashi (P = 1.7e-71)</td>
<td>Cell-cell signaling (GO:0007267), nuclear mRNA splicing via spliceosome (GO:0000398), oogenesis (GO:0048477), 11 other terms</td>
</tr>
</tbody>
</table>

*Results generated using AmiGO (1) gene ontology database release 2010-11-20.
†Note that the low number of substitutions in this gene severely limits the inference of positive selection.

Table S2. Genes under positive selection as determined by branch-site analysis

<table>
<thead>
<tr>
<th>S. invicta gene ID</th>
<th>Branch-site MUSCLE P value</th>
<th>Branch-site PRANK P value</th>
<th>&gt;Twofold expression</th>
<th>AmiGO* BLAST protein similarity</th>
<th>Gene ontology biological process</th>
</tr>
</thead>
<tbody>
<tr>
<td>SI2.2.0_02629</td>
<td>1.88e-11</td>
<td>1.19e-11</td>
<td>Male adult up-regulation vs. queen adult, male pupa up-regulation vs. queen pupa</td>
<td>No hits (P &lt; 1e-5)</td>
<td></td>
</tr>
<tr>
<td>SI2.2.0_05545</td>
<td>9.09e-7</td>
<td>8.07e-7</td>
<td>Male pupa up-regulation vs. queen pupa</td>
<td>Drosophila melanogaster Bifunctional phosphopantetheine adenylyltransferase-dephospho-CoA kinase (P = 1.5e-95)</td>
<td>CoA biosynthetic process (GO:00015937), imaginal disk-derived wing morphogenesis (GO:0007476), ovarian follicle cell migration (GO:0007297), two other terms</td>
</tr>
<tr>
<td>SI2.2.0_11797</td>
<td>4.39e-6</td>
<td>1.94e-5</td>
<td>None</td>
<td>Gallus gallus Uncharacterized protein (P = 3.6e-249)</td>
<td>Nucleobase, nucleoside, nucleotide, and nucleic acid metabolic process (GO:0006139)</td>
</tr>
<tr>
<td>SI2.2.0_12264</td>
<td>4.90e-10</td>
<td>1.37e-4</td>
<td>None</td>
<td>Pan troglodytes Dyslexia susceptibility 1 candidate gene 1 protein homolog (P = 8.7e-56)</td>
<td>Neuron migration (GO:0001764), regulation of estrogen receptor signaling pathway (GO:0033146), regulation of proteasomal protein catabolic process (GO:0066136)</td>
</tr>
</tbody>
</table>

*Results generated using AmiGO (1) gene ontology database release 2010-12-04.


<table>
<thead>
<tr>
<th>Category</th>
<th>No. genes</th>
<th>Direction of selection*</th>
<th>Dn/s</th>
<th>Ds/s</th>
<th>Pn</th>
<th>P1</th>
<th>P2/D1</th>
<th>P3/Ps</th>
<th>P value†</th>
</tr>
</thead>
<tbody>
<tr>
<td>Less than 1.25-fold bias in all comparisons</td>
<td>23</td>
<td>-0.128</td>
<td>564</td>
<td>2,816</td>
<td>11</td>
<td>26</td>
<td>0.200</td>
<td>0.423</td>
<td>0.054</td>
</tr>
<tr>
<td>Less than 1.5-fold bias in all comparisons</td>
<td>99</td>
<td>-0.125</td>
<td>2,139</td>
<td>11,323</td>
<td>48</td>
<td>120</td>
<td>0.189</td>
<td>0.400</td>
<td>4.3e-5</td>
</tr>
<tr>
<td>Less than twofold bias in all comparisons</td>
<td>223</td>
<td>-0.126</td>
<td>5,814</td>
<td>24,779</td>
<td>124</td>
<td>268</td>
<td>0.325</td>
<td>0.463</td>
<td>3.4e-9</td>
</tr>
<tr>
<td>Greater than twofold bias in one or more of five comparisons</td>
<td>158</td>
<td>-0.050</td>
<td>5,752</td>
<td>18,639</td>
<td>92</td>
<td>209</td>
<td>0.342</td>
<td>0.440</td>
<td>0.048</td>
</tr>
<tr>
<td>Greater than twofold bias between adult sexes</td>
<td>34</td>
<td>0.004</td>
<td>1,783</td>
<td>3,701</td>
<td>20</td>
<td>43</td>
<td>0.482</td>
<td>0.465</td>
<td>0.898</td>
</tr>
<tr>
<td>Greater than twofold bias between pupal sexes</td>
<td>80</td>
<td>-0.024</td>
<td>3,919</td>
<td>8,669</td>
<td>52</td>
<td>102</td>
<td>0.452</td>
<td>0.510</td>
<td>0.487</td>
</tr>
<tr>
<td>Greater than twofold bias between adult castes</td>
<td>18</td>
<td>-0.005</td>
<td>668</td>
<td>1,938</td>
<td>11</td>
<td>31</td>
<td>0.345</td>
<td>0.355</td>
<td>0.935</td>
</tr>
<tr>
<td>Greater than twofold bias between pupal castes</td>
<td>12</td>
<td>-0.039</td>
<td>357</td>
<td>807</td>
<td>7</td>
<td>13</td>
<td>0.442</td>
<td>0.538</td>
<td>0.685</td>
</tr>
<tr>
<td>Greater than twofold bias between developmental stages</td>
<td>100</td>
<td>-0.050</td>
<td>3,050</td>
<td>10,156</td>
<td>54</td>
<td>137</td>
<td>0.300</td>
<td>0.394</td>
<td>0.101</td>
</tr>
</tbody>
</table>

*Direction of selection [Dn/(Ds + Dn) − Pn/(Pn + P1)] is calculated according to Stoletzki and Eyre-Walker (1), where a negative value indicates purifying selection and a positive value indicates positive selection.

1 Dn represents the total number of synonymous fixed differences, Pn represents the total number of synonymous polymorphisms, Ds represents the total number of nonsynonymous fixed differences, and Ps represents the total number of nonsynonymous polymorphisms (Methods).

†P value denotes the results of the McDonald–Kreitman (2) test according to a G test of independence (with the Williams correction for continuity).


Table S4. Associations between S. invicta codon usage, CpG depletion, expression level, and evolutionary rates

<table>
<thead>
<tr>
<th>Principal component †</th>
<th>1</th>
<th>2</th>
<th>3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Percent variance explained in CpG o/e, expression level, Fop, and GC3s</td>
<td>60.923</td>
<td>24.715</td>
<td>14.265</td>
</tr>
<tr>
<td>Rank correlation with S. invicta dN/dS</td>
<td>-0.02</td>
<td>-0.19****</td>
<td>0.19****</td>
</tr>
<tr>
<td>Rank correlation with S. invicta dN</td>
<td>-0.09**</td>
<td>-0.17****</td>
<td>0.18****</td>
</tr>
<tr>
<td>Rank correlation with S. invicta dS</td>
<td>-0.17****</td>
<td>0.03</td>
<td>0.01</td>
</tr>
<tr>
<td>Percent contributions to principal component</td>
<td>22.4</td>
<td>2.7</td>
<td>74.9</td>
</tr>
<tr>
<td>CpG o/e</td>
<td>1.3</td>
<td>97.1</td>
<td>1.6</td>
</tr>
<tr>
<td>Expression level</td>
<td>38.2</td>
<td>0.1</td>
<td>11.8</td>
</tr>
<tr>
<td>GC3s</td>
<td>38.2</td>
<td>0.1</td>
<td>11.7</td>
</tr>
</tbody>
</table>

†Principal component 4 explains 0.10% of the variance in CpG o/e, expression level, Fop, and GC3s, and it is not presented. CpG o/e, normalized CpG dinucleotide content; GC3s, third codon position synonymous GC content.

**P < 0.01; ****P < 0.0001.